In vitro Cellular Immune Reactions of Hemocytes against Bacteria and their Differential Degradation in Myriapods

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ABSTRACT

In vitro the hemocytes of various diplopods and chilopods are capable of phagocytosis and degradation of bacteria. The sequence of this process depends on the myriapod species and the type of bacteria. In *Rhapidostreptus virgator* many *Micrococcus luteus* have been phagocytosed but occur uneffected after 4 hours whereas *E. coli* shows indications of lysis. After 20 hours *M. luteus* also has been degradated. In *Lithobius forficatus*, nodules, aggregations of hemocytes phagocytosing bacteria, are formed when hemocytes are added to a bacterial culture which is turned around continuously. During this process, the hemocytes degranulate. *M. luteus* is lysed within 1 hour when phagocytosed or even located close to a hemocyte cell. *Enterobacter cloacae* is enclosed extracellulary within the matrix of the nodule and subsequently phagocytosed. *E. coli* was not aggregated within the nodule but hemocytes discharged vesicular content onto their surface. They become phagocytosed but do not show indications of lysis after 1 hour. In *Scolopendra cingulata* hemocytes also form nodules with bacteria in the continuously moved culture. These nodules contain a large extracellular matrix in which the bacteria are embedded. Only very few bacteria are phagocytosed within 1h of incubation and there are no signs of lysis in *M. luteus*.

RÉSUMÉ

Réactions immunitaires in vitro d'hémocytes contre des bactéries et leur dégradation chez les myriapodes (Diplopoda & Chilopoda).

In vitro, les hémocytes de différents diplopodes et chilopodes sont capables de phagocyter et de dégrader des bactéries. La séquence de ces processus dépend des espèces de myriapodes et de bactéries. Au bout de 4 heures, chez *Rhapidostreptus virgator*, de nombreux *Micrococcus luteus* sont phagocytés mais ne subissent aucun dommage, tandis que *E. coli* montre déjà des indices de lyse. Au bout de 20 heures, *M. luteus* est, lui aussi, dégradé. Chez *Lithobius forficatus*, de petits nodules se forment, agrégations d'hémocytes phagocytant des bactéries, si des hémocytes sont ajoutés à une culture bactérienne continuellement remuée. Durant ce processus, les hémocytes dégranulent. *M. luteus* est lysé en 1 heure, si les bactéries sont phagocytées ou même seulement se trouvent à proximité d'un hémocyte. *Enterobacter cloacae* se trouve inclus de manière extracellulaire dans la matrice du nodule, puis phagocyté. *E. coli* ne montre pas d'agrégation en nodules, mais les hémocytes déchargent le contenu de certaines vésicules sur la surface bactérienne. Elles seront finalement phagocytées mais aucune trace de lyse n'est visible au bout de 1 heure. Chez *Scolopendra cingulata*, les hémocytes forment des nodules avec les bactéries en culture. Ces nodules contiennent une volumineuse matrice extracellulaire dans laquelle les bactéries sont incorporées. Au bout de 1 heure, seul un très petit nombre d'entre elles est phagocyté ou lysé.

NEVERMANN, L. & XYLANDER, W. E. R., 1996. — In vitro cellular immune reactions of hemocytes against bacteria and their differential degradation in myriapods. *In*: GEOFFROY, J.-J., MAURIÈS, J.-P. & NGUYEN DUY - JACQUEMIN, M., (eds), Acta Myriapodologica. *Mém. Mus. natn. Hist. nat.*, **169** : 421-430. Paris ISBN : 2-85653-502-X.

INTRODUCTION

Two mechanisms are involved in defense of arthropods against infections by microorganisms: the formation of antibacterial substances which in insects mainly act free in the plasma, and the action of defense cells e. g. hemocytes which form nodules or phagocytose the microbes. Lysozyme, an enzyme depolymerizing the cell wall of Gram-positive bacteria, is permanently found in the hemolymph (DUNN, 1986; GÖTZ, 1988) whereas most antibacterial substances against Gram-positive bacteria are formed within a few hours after an external stimulus, e. g. infections or injury (cf. BOMAN, 1986; GÖTZ, 1988). Therefore, nodule formation and phagocytosis are the initial defense mechanisms against microbes that help to absorb infective bacteria until other mechanisms are available.

The mechanisms and the procedure of cellular defense against bacteria has been well documented in insects, whereas little is known about such processes in "myriapods". This paper, therefore, describes nodule formation, phagocytosis, specificity of phagocytic hemocytes to different bacteria and degradation of ingested bacteria in the diplopod *Rhapidostreptus virgator* and the chilopods *Lithobius forficatus* and *Scolopendra cingulata*.

MATERIAL AND METHODS

Animals

Specimens of *Scolopendra cingulata* were collected in 1990 at different sites in northern Spain and maintained solitary since then in large bellaplast boxes with 2 cm of clean sand or soil covered with paper towels at room temperature at a shaded place. Humidity of the substratum was controlled weekly and moistened with tab water if necessary. Specimens were fed with *Acheta domesticus*, *Tenebrio molitor* mainly larvae and *Sarcophaga* sp. mainly imagines. *Lithobius* and *Rhapidostreptus* were obtained and reared as described by XYLANDER & NEVERMANN (1990). Hemolymph was obtained as specified earlier (XYLANDER & NEVERMANN, 1990; XYLANDER & BOGUSCH, 1992) and dropped directly into the culture medium.

Hemocyte preparations

For investigations with hemocytes of *Rhapidostreptus* hemolymph was added to a 1 cm^2 piece of gelatine foil after swelling it in I-Ringer (11 g NaCl, 7 g KCl, 5.5 g $CaCl_2$, in 1l H_2O) for 30 min in culture dishes. Hemocytes were allowed to adhere to the substratum in I-Ringer for 50 min at room temperature. Then 10 µl of a bacterial suspension were added. Hemocytes cultures with bacteria were slowly moved on a shaker for 1 h, 4 h and 20 h.

For investigations on chilopods 10 µl of suspended "washed" bacteria (see below) were added to 1 ml LAH (*Lithobius*-artificial-hemolymph according to WENNING,1989) in an Eppendorf cap and then 10 µl freshly collected hemolymph was supplied. The Eppendorf caps were fixed with cello tape to a spinbar and rotated vertically on a magnetic stirrer for 1 h at room temperature. Subsequently, the Eppendorf cap was centrifuged at 100 x g for about a minute, the supernatant was removed and the pellet was processed for electron microscopy.

Preparation of bacterial cultures

The bacteria used in this study were obtained from Prof. Dr. P. GÖTZ, Berlin (*Micrococcus luteus*, *Enterobacter cloacae* ß-12 and *Escherichia coli* K12 D31). Bacteria were cultivated in nutrient broth (Merck Standard I) over night at 35°C in a water bath. Small amounts of this "over-night-culture" were added to fresh broth and raised until optical density reached 0.65 at 565 nm.

For investigations on chilopods 1 ml of bacteria in culture were washed twice by centrifugation (room temperature, 5 min at 1000 x g) and resuspension of the bacterial pellet to 1 ml with LAH. Finally, 10 µl of the resuspension were added to the hemocyte culture.

Preparation for transmission electron microscopy

All samples were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.0 for 2 h at 4-6°C, washed in buffer, postfixed in 2% OsO_4, dehydrated through an acetone series and embedded in araldite. Semithin and ultrathin sections were made on a Reichert OmU3 ultracut microtome, mounted on formvar coated copper grids, stained with uranyl acetate and lead citrate and investigated with a Zeiss EM 9 A transmission electron microscope (TEM).

RESULTS

Rhapidostreptus

Within 4 h after addition of bacteria the hemocytes of *Rhapidostreptus* have phagocytosed both Gram-negative *E. coli* and Gram-positive *Micrococcus luteus* (Figs 1, 2, 5 & 6). The bacteria are located in vacuoles mainly in that part of the hemocytes without contact to the

substratum (Figs 1 & 5); the vacuoles have been found to be smaller around *Micrococcus* than around *E. coli* (Figs 1 & 5). Most *Micrococcus* remain rather uneffected and still show their typical electron dense internal structure (Figs 1 & 2) whereas the inside of *E. coli* occurs less electron dense and flocculent indicating degradation (Figs 5 & 6). After 20 h dramatic changes are found. The cell wall of phagocytosed *Micrococcus* has been completely degradated and the bacteria have been killed as noticed from an obvious decrease in their electron density (Figs 3 & 4). Plasmatocytes of *Rhapidostreptus* have disintegrated after 20 h leaving a translucent vesicular cell debris whereas granulocytes look relatively unchanged. Bacteria which have not been phagocytosed still exhibit their normal structure but show electron dense amorphous material at their surface.

Lithobius forficatus

After 1 h in the "stirrer culture" plasmatocytes, granulocytes and some spherulocytes (for description and classification of hemocytes, see NEVERMANN *et al.*, 1991) have aggregated to form nodules (Fig. 7). Plasmatocytes and granulocytes, however, no longer can be differentiated, since they have lost most of their grana obviously by exocytosis into the culture medium. All three species of bacteria are phagocytosed. Phagocytic vesicles enclosing bacteria have been observed to fuse with lysosomes and become electron dense (Figs 8, 9, 11 & 16).

Micrococcus is lysed in electron translucent phagocytic vesicles (Fig. 14). Their cell wall is thinned off and their plasm becomes more electron translucent in TEM compared to living bacteria (Figs 14 & 15). Even bacteria attached to the surface of hemocytes or lying in short distance to hemocytes frequently undergo lysis. Bacteria, located in some distance are uneffected (Fig. 15).

Enterobacter, in contrast to *Micrococcus* and *E. coli*, becomes aggregated in the hemolymph culture and, therefore, clusters of bacteria are surrounded by hemocytes (Fig. 7). Nevertheless, some of the bacteria are phagocytosed and lysed (Figs 7-9). In one single phagocytic vesicle different stages of bacterial lysis may be found. Destroyed bacteria swell and occur less electron dense (Figs 7 & 8).

E. coli are not aggregated but show a scattered distribution throughout the hemocyte nodule. They become phagocytosed occasionally but no indications of lysis was found after 1 h of culture neither in translucent nor in electron dense phagocytic vesicles (Figs 10-13). Prior to phagocytosis fibrous material from the so-called structured vesicles (see NEVERMANN *et al.*, 1991) is discharged onto the surface of *E. coli* (Fig. 10). The same material may also be found in phagocytic vesicles containing bacteria (Fig. 12).

Scolopendra cingulata

The hemocytes of *S. cingulata* aggregate to form nodules which contain an voluminous, possibly fibrous, extracellular material (Fig. 17). Many bacteria (only *Micrococcus* was tested) are embedded in this matrix and most of them seem to have no contact to the hemocytes. Nevertheless, some bacteria are phagocytosed. As in *Lithobius* the phagocytic vesicles of *Scolopendra* may have an electron dense matrix but - in contrast to *Lithobius* - *Micrococcus* does not get lysed (Fig. 16). Presumably, in this species it takes more time than 1 h until visible signs of lysis are found in TEM.

DISCUSSION

Phagocytosis has been described for insects in a number of papers but for other groups of arthropods there is only very little information on phagocytosis and intracellular degradation of microbes by hemocytes (e. g. JOHNSON, 1981; PALM, 1953; TYSON & JENKIN, 1973). The TEM-micrographs of phagocytosis in arthropods, however, are very similar indicating that the

mechanisms must be quite alike. Even intracellular degradation of bacteria and the appearance of electron-dense phagocytic vesicles are much alike in insects (cf. ROWLEY & RATCLIFFE, 1976a and our investigation on chilopods).

Two initial mechanisms are involved in host cellular defense reaction against invading bacteria: phagocytosis and nodule formation. Phagocytosis is supposed to be the main reaction against low numbers of bacteria in arthropods (GÖTZ, 1982; CHRISTENSEN & NAPPI, 1988). But when there are high numbers of microbes entering the hemocoel nodule formation occurs (RATCLIFFE & GAGEN, 1977). Our in vitro observations of *Lithobius* and *Scolopendra* indicate that nodule formation and phagocytosis are related processes in the coagulum.

In the spiny lobster, *Panulirus japonicus* a factor is released from granular hemocytes after stimulation with bacteria which provokes clotting of the other hemocyte types (AONO *et al.*, 1993). This implies that even a small number of microbes can elicit clotting. Nodule formation may, therefore, not be restricted to reactions against higher numbers of bacteria but is a general process occurring parallel to phagocytosis.

Lithobius hemocytes can degradate bacteria faster (in only 1 h) than *Scolopendra* and *Rhapidostreptus* and other arthropods investigated. In an *in vitro* investigation with hemocytes of *Calliphora erythrocephala* ROWLEY & RATCLIFFE (1976a) found cell wall damage and swollen bacteria not before 2 h of incubation. Heat killed bacteria *Bacillus cereus* injected into the hemocoel were entrapped into nodules but they did not show any signs of breakdown even after 24 h (RATCLIFFE & GAGEN, 1977).

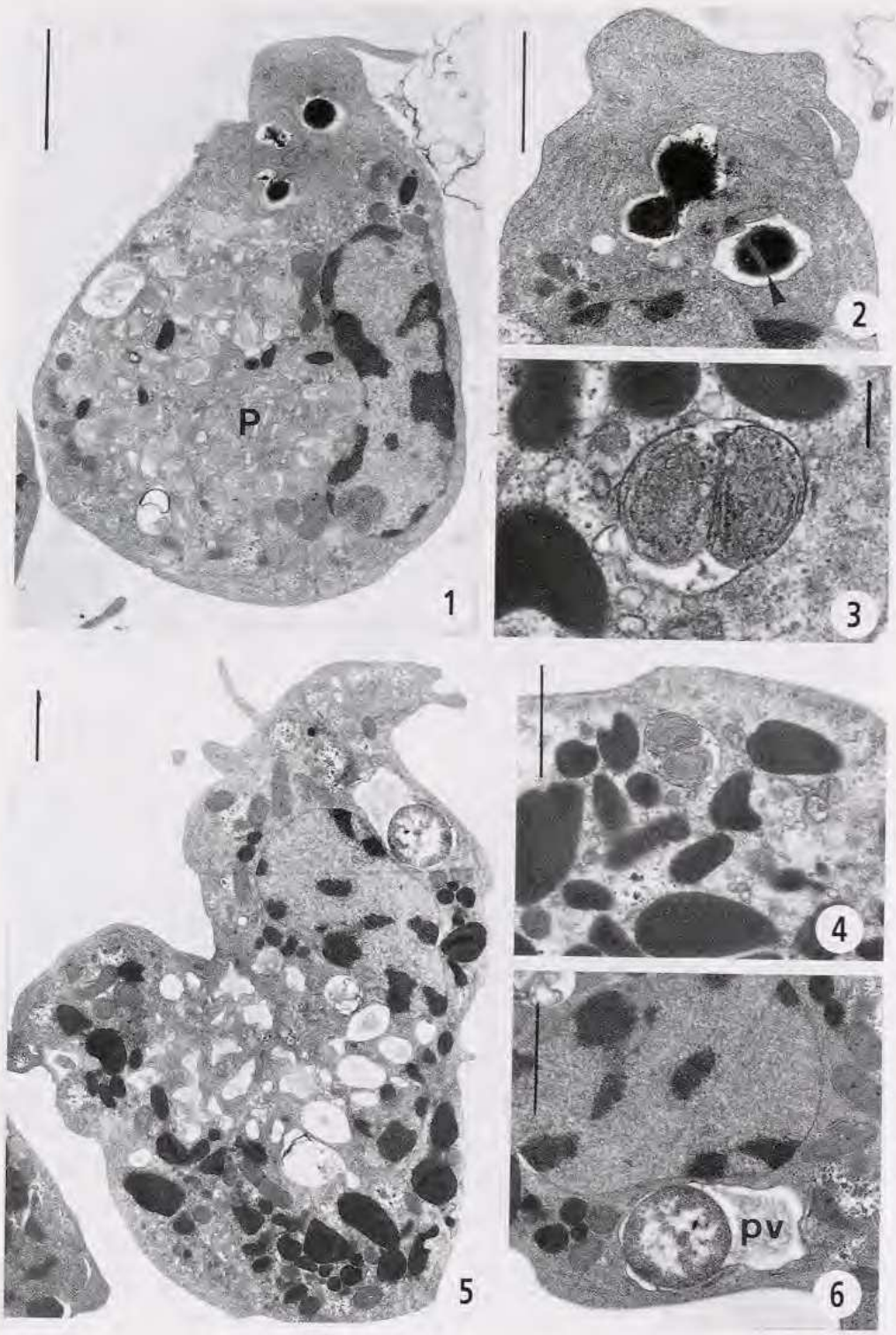
The reaction of hemocyte types to different bacteria varies. Granular cells of *Galleria mellonella* make contact with *E. coli* in vitro, get stressed and degranulate (ROWLEY & RATCLIFFE, 1976b). Eventually, they may phagocytose *E. coli* but most bacteria remain attached to the outside of the cells. The plasmatocytes of this species remain unstressed and phagocytose bacteria and the decaying granular cells. However, no killed bacteria have been observed. In this investigation all hemocyte types were involved in phagocytosis and nodule formation. In *Rhapidostreptus* granular hemocytes even seemed to be more stable and less sensible to lysis than plasmatocytes.

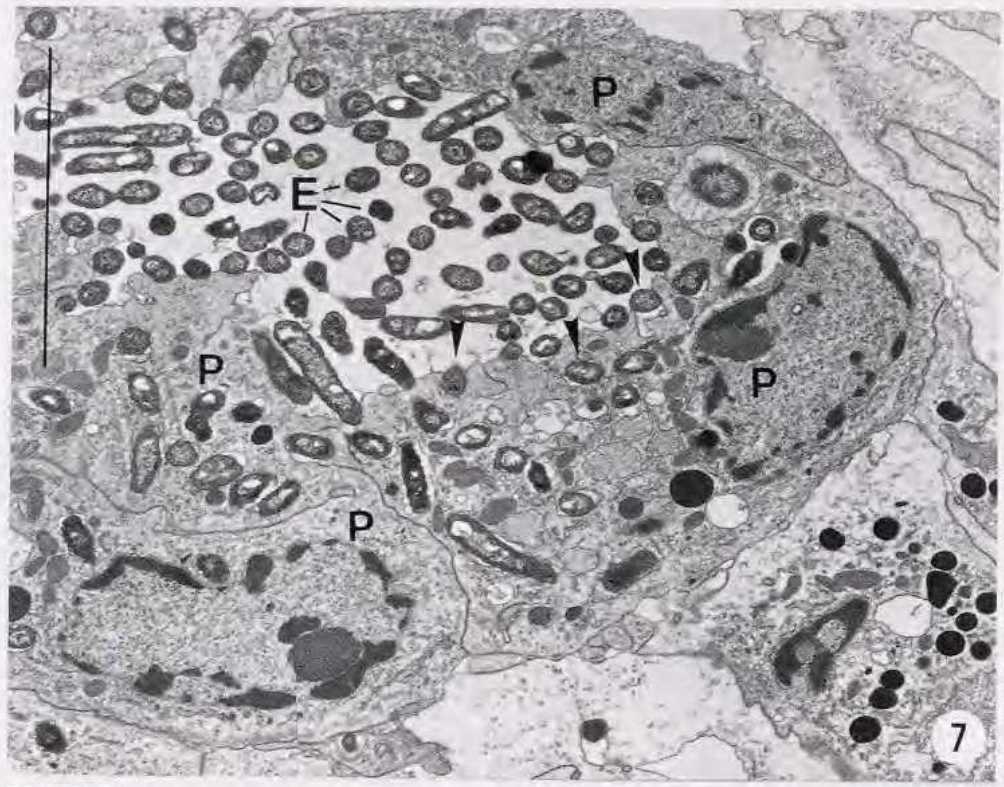
FIGS 1-6. — *Rhapidostreptus virgator*: 1. Plasmatocyte (P) containing some not lysed *Micrococcus luteus* in phagocytic vesicles, 4 h of incubation. Scale bar: 2 µm. 2. Plasmatocyte with phagocytosed *M. luteus*, 4 h of incubation. The diplococcal structure of the bacteria is visible (arrowhead). Scale bar: 1 µm. 3. Granulocyte (II) with phagocytosed *M. luteus* which has become translucent due to lysis, 20 h of incubation. Scale bar: 0.2 µm. 4. Granulocyte (II) with phagocytosed lysed *M. luteus*, 20 h of incubation. Scale bar: 1 µm. 5. Granulocyte (I) with phagocytosed and lysed *E. coli*, 4 h of incubation. The phagocytic vesicle surrounding the bacterium is significantly larger than that around *M. luteus*. Scale bar: 1 µm. 6. Granulocyte (I) with lysed *E. coli* in the phagocytic vesicle (pv) showing a flocculent less electron dense content, 4 h of incubation. Scale bar: 1 µm.

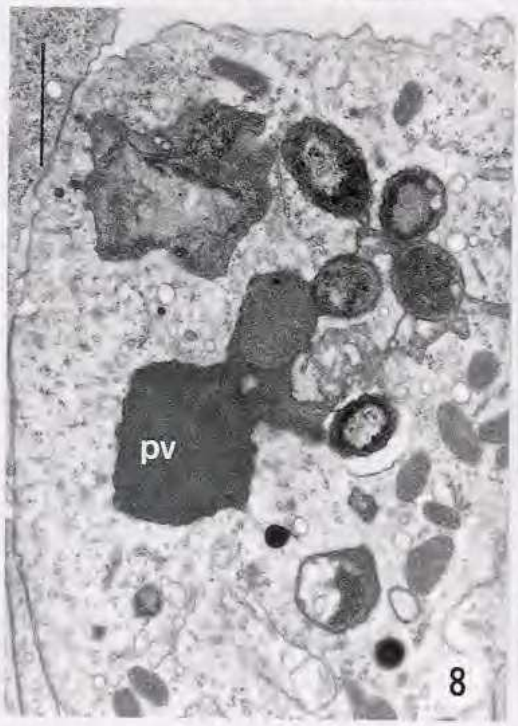
FIGS 7-9. — *Lithobius forficatus*: 7. Hemocytic nodule. Plasmatocytes (P) forming a primary capsule around agglutinated *Enterobacter cloacae* (E). Arrowheads indicate bacteria just being phagocytosed. Scale bar: 5 µm. 8. Large electron dense phagocytic vesicle (pv) containing *E. cloacae* in various stages of degradation. Scale bar: 1 µm. 9. Fusion of an electron dense lysosome with a phagocytic vesicle containing *E. cloacae*. Scale bar: 0.5 µm.

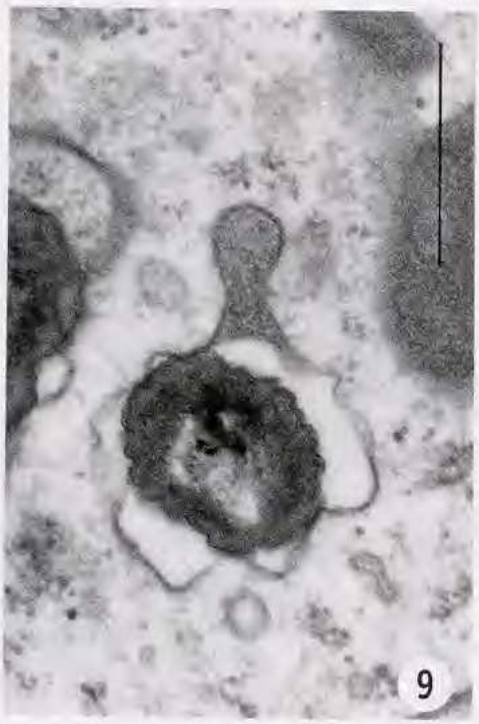
FIGS 10-13. — *Lithobius forficatus*: 10. *E. coli* during phagocytosis. Note fibrous material (*) discharged onto bacteria. 11. Electron dense phagocytic vesicle (pv) with five *E. coli* without clear indications of lysis. 12. Phagocytic vesicle of a granular hemocyte with structured content (sv) and phagocytosed *E. coli*. The cell shows signs of disintegration. 13. Small translucent vesicles (arrow) gathering around phagocytosed *E. coli*. All scale bars on this plate: 1 µm.

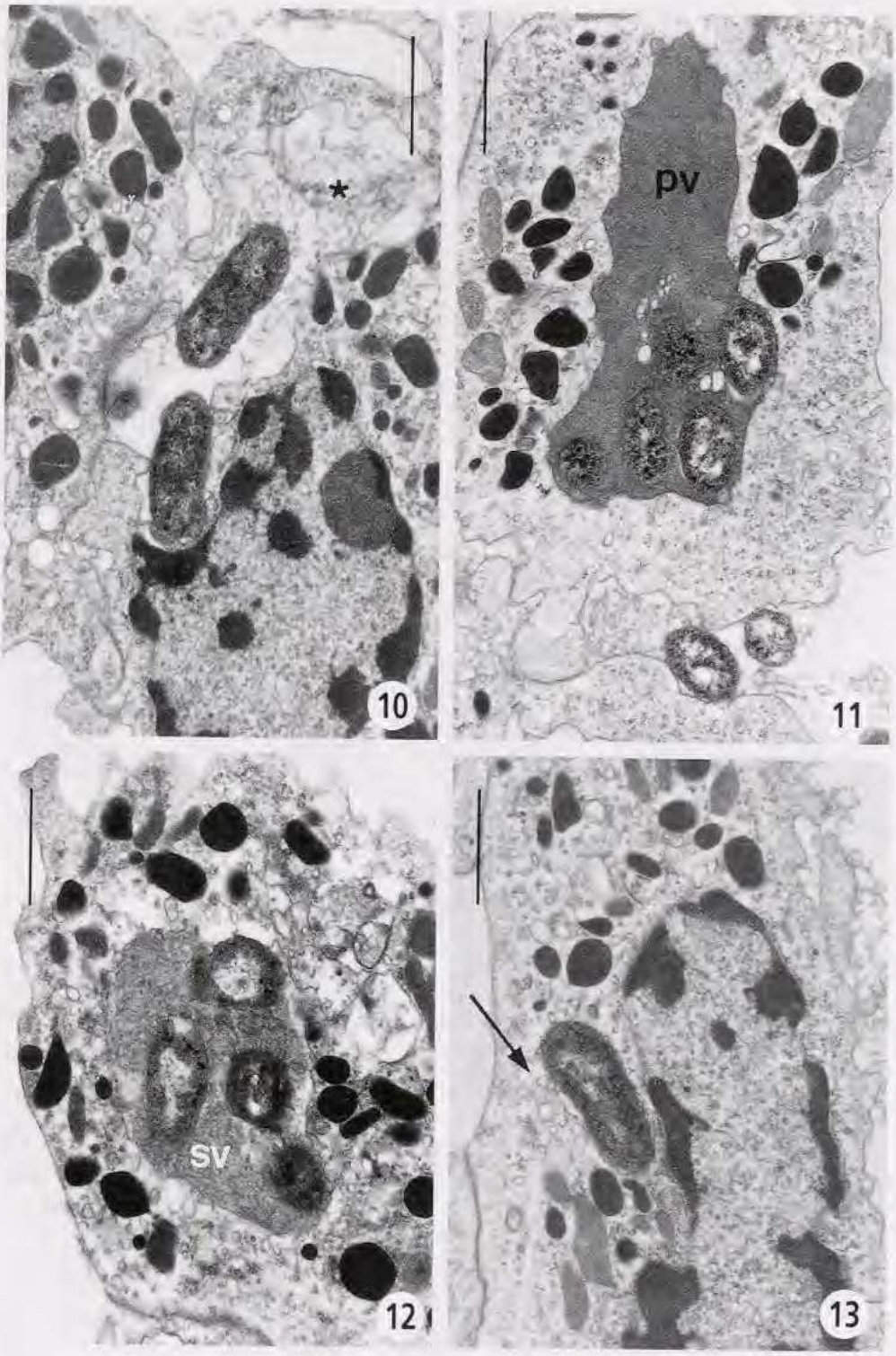
FIGS 14-15. — *Lithobius forficatus* & FIGS 16-17.— *Scolopendra cingulata*: 14. Phagocytosed and lysed *Micrococcus luteus* (M). Note the thinned murein sacculus. The hemocytes have lost most of their grana. Scale bar: 5 µm. 15. Few still electron dense *M. luteus* with an intact murein sacculus (arrow) at some distance to a hemocyte. Bacteria located closer are more electron translucent on lysis. Arrowhead: semi-lysed diplococcus. Scale bar: 1 µm. 16. Four *M. luteus* in an electron dense phagocytic vesicle (pv) without signs of degradation. Scale bar: 1 µm. 17. Nodules of *S. cingulata* with an voluminous extracellular matrix (X) embedding the hemocytes and *M. luteus* (M). Some bacteria are being phagocytosed (Mp). Scale bar: 1 µm.

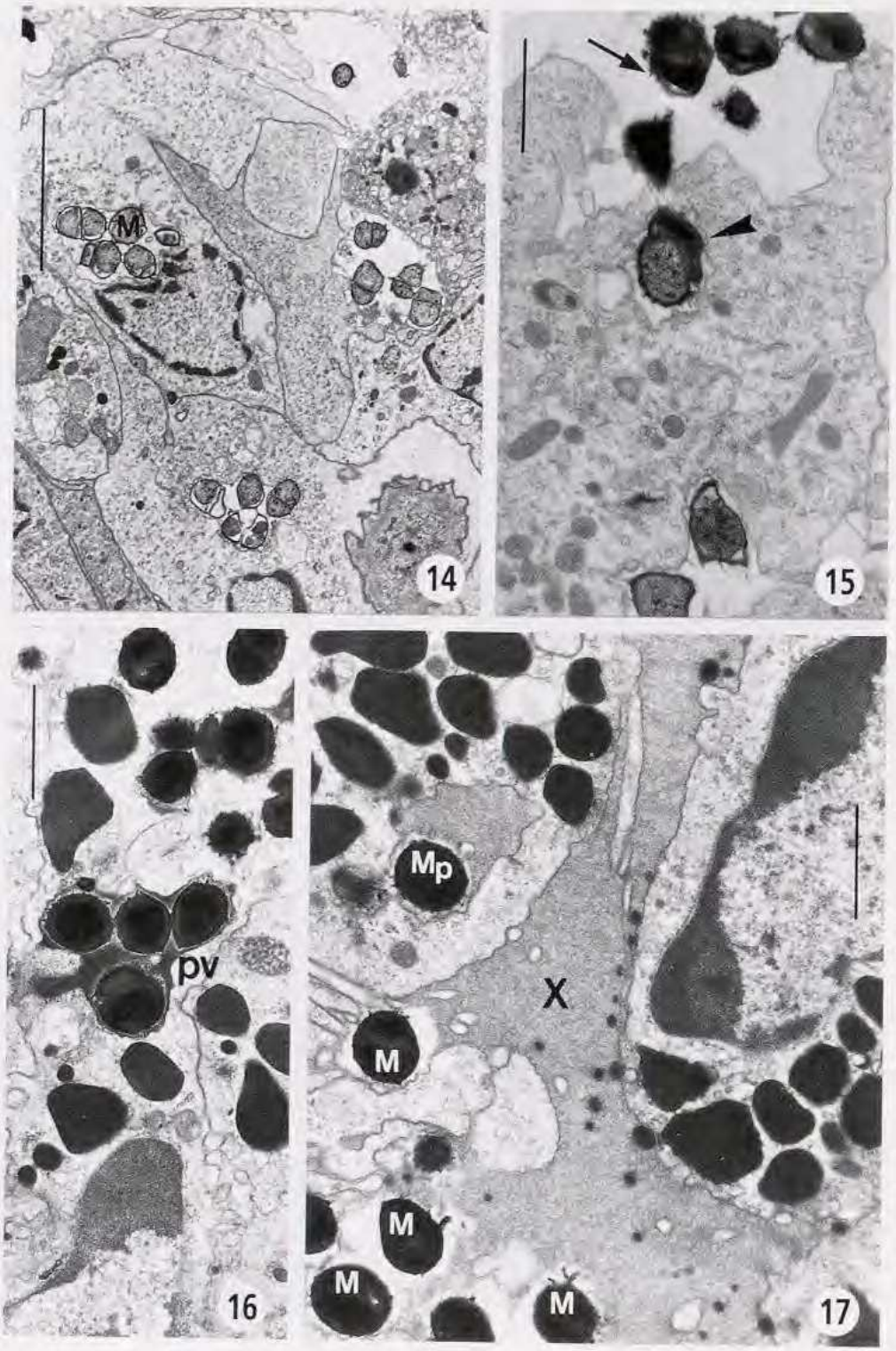












The time span until bacterial degradation becomes visible differs in the species investigated and may be due to antibacterial substances which have earlier been demonstrated by bacterial-agar diffusion tests in various diplopods and chilopods (VAN DER WALT *et al.*, 1990; XYLANDER & NEVERMANN, 1990). Antibacterial substances produced by hemocytes may be involved in the intracellular and extracellular degradation of bacteria as observed. The thinning of the cell wall of *Micrococcus luteus* indicates the action of lysozym which also has been shown to occur in the hemolymph of various diplopods and chilopods (XYLANDER & NEVERMANN, 1990). In the investigations by XYLANDER & NEVERMANN (1990), *Scolopendra oraniensis* and *Rhapidostreptus* had less effect on living bacteria than e. g. *Lithobius*; especially the lysozyme effect on lyophilized *Micrococcus luteus* was low. Thus, the delay in cell wall lysis in *Scolopendra* and *Rhapidostreptus* may be caused by lower titers of lysozym available in the hemocytes. This corresponds to recent immunocytochemical detection of varying amounts of lysozyme in hemocytes of chilopods (NEVERMANN, unpublished).

In many insects investigated the fat body is the main site of synthesis of antibacterial substances after infections; they are discharged into the hemolymph where they destroy bacteria. Hemocytes may also produce such substances (TRENCZEK, 1988) but their importance in insects for humoral antibacterial defense seems to be low. However, in other arthropods like crustaceans (FENOUIL & ROCH, 1991; SMITH & CHRISHOLM, 1992), xiphosurans (MURAKAMI *et al.*, 1991; NAKAMURA *et al.*, 1988; TOH *et al.*, 1991), diplopods (XYLANDER, unpublished) and chilopods (NEVERMANN, unpublished), the antibacterial substances are mainly located in the hemocytes which most probably also are the site of their formation. Located in the hemocytes the antibacterial substances may be strongly involved in killing and subsequent or simultaneous lysis of bacteria. This could probably be the original function (and site of formation) of the antibacterial substances in arthropods; in insects or a subgroup of this taxon the fat body seems to have taken over this function from hemocytes. Thereby, the antibacterial defense may have become subdivided in an initial cellular and a subsequent humoral phase.

However, further proteins may be involved in the cellular defense against bacteria. Agglutination of bacteria as found in *Lithobius* could be due to lectins which have been found in the hemolymph plasma of different diplopods (JAHN & SEIFERT, 1992; JAHN & XYLANDER, 1991; XYLANDER, 1990, 1992) and may - as shown in e. g. insects (PENDLAND *et al.*, 1988) - be responsible for bacterial agglutination and opsonization facilitating phagocytosis.

ACKNOWLEDGEMENTS

We would like to thank Miss A. HUDEL for printing the micrographs and the President of the Justus-Liebig-University, Giessen, who supported this investigation and our participation in the congress.

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